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Enzyme-Linked Sandwich Immunoassay of Macromolecular Antigens Using the Rabbit Antibody-Coupled Glass Rod as a Solid Phase

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A highly sensitive sandwich immunoassay of macromolecular antigens using the rabbit antibody Fab'–β-D-galactosidase complex and the rabbit antibody immunoglobulin-G-coupled glass rod as a solid phase is described. The Fab' fragments of rabbit antibody IgG are conjugated with β-D-galactosidase from *Escherichia coli* using *N,N'-o*-phenylenedimaleimide. Rabbit antibody IgG is coupled to the aminoalkylsilyl glass rods (3 mm in diameter and 5 mm in length) using glutaraldehyde. A wide range of the concentrations of rabbit IgG fraction (20–2000 μg/ml) is effective for coupling, and the amount of rabbit immunoglobulin G coupled can be controlled. The smallest amounts of ornithine δ-aminotransferase from rat liver, human immunoglobulin G and 2,4-dinitrophenyl human immunoglobulin G that can be determined are 0.03, 0.3 and 0.04 fmol, respectively. The sensitivity of the assay for these antigens is affected mainly by the non-specific binding of the complexes to the solid phase and the ability of antigen molecules, adsorbed on the solid phase, to bind specifically the complexes. The assay with the rabbit antibody immunoglobulin-G-coupled glass rods is simpler and more sensitive than that with the rabbit antibody immunoglobulin-G-coupled Sepharose 4B.

Several procedures have been developed for the enzyme-linked solid phase sandwich immunoassay of macromolecular antigens [1–5]. Enzymes that have been used for labelling antibodies are alkaline phosphatase [1], peroxidase [2], glucose oxidase [3] and β-D-galactosidase [4, 5]. Antibodies have been labelled with enzymes using glutaraldehyde [1–3] or *N,N'-o*-phenylenedimaleimide [4, 5]. Solid phases that have been used are polystylene tubes [1, 2], cellulose [3] and Sepharose [4, 5].

We have recently developed a novel method for the conjugation of Fab' fragments of rabbit IgG with β-D-galactosidase from *Escherichia coli* and have demonstrated that 0.3 fmol of human IgG are measurable using the purified rabbit anti(human IgG) Fab'–β-D-galactosidase complex and the rabbit anti-(human IgG) IgG-coupled Sepharose 4B [6, 7]. The present paper describes a highly sensitive sandwich immunoassay of macromolecular antigens using the rabbit antibody Fab'–β-D-galactosidase complex and the rabbit antibody IgG-coupled glass rod as a solid phase.

Abbreviations. IgG, immunoglobulin G; Fab', monovalent fragments derived from pepsin-treated IgG; buffer A, 0.01 M sodium phosphate buffer (pH 7.0) containing 0.1 M NaCl, 1 mM $MgCl_2$, 0.1% NaN_3 and 0.1% bovine serum albumin.

Enzymes. Alkaline phosphatase (EC 3.1.3.1); peroxidase (EC 1.11.1.7); glucose oxidase (EC 1.1.3.4); β-D-galactosidase (EC 3.2.1.23); L-ornithine : 2-oxo-acid aminotransferase or ornithine δ-aminotransferase (EC 2.6.1.13).

MATERIALS AND METHODS

Antigens and Antibodies

Crystalline ornithine δ-aminotransferase from rat liver and rabbit antisera against the crystalline enzyme were prepared as described previously [8]. The preparation of the crystalline enzyme was homogeneous on ultracentrifugation and electrophoresis and could be stored in 0.1 M sodium phosphate buffer (pH 7.5) without loss of its activity at −20 °C for at least 6 months. Amounts in μg of the crystalline enzyme were determined by the method of Lowry *et al.* [9] using bovine serum albumin (fraction V, Armour Pharmaceutical Co., Chicago) as a standard and its amounts in fmol were calculated using its molecular weight of 170000 [8]. The IgG fraction from the antiserum against the enzyme was obtained by fractionation with $(NH_4)_2SO_4$ [10] followed by passage through a column of DEAE-cellulose (DE 52 Whatman Biochemicals Ltd, Kent) [11]. Human IgG was

obtained from Miles Laboratories, Inc. (Kankakee) and its amounts were determined from the absorbance at 280 nm [12]. 2,4-Dinitrophenyl human IgG was prepared using 2,4-dinitrobenzene sulfonic acid (sodium salt) (Tokyo Kasei Kogyo Co., Ltd, Tokyo) by the method of Eisen *et al.* [13]. The number of 2,4-dinitrophenyl residues coupled to human IgG molecules and the amount of 2,4-dinitrophenyl human IgG were calculated from the absorbance at 280 and 360 nm [12, 13]. The rabbit antisera against human IgG and 2,4-dinitrophenyl bovine serum albumin were obtained from Medical and Biological Laboratories Ltd (Nagoya) and from Miles Labotatories, Inc. (Kankakee), respectively. The IgG fractions from these antisera and non-specific rabbit serum were prepared using Na_2SO_4 [14] and DEAE-cellulose [11].

The Rabbit IgG-Coupled Glass Rods

The rabbit IgG-coupled glass rods were prepared by the method of Robinson *et al.* [15]. Pyrex glass rods (3 mm in diameter and 5 mm in length) were heated at 500 °C for 5 h and then were immersed in 2% 3-aminopropyltriethoxysilane (Nakarai Chemicals Ltd, Kyoto) in acetone at 45 °C for 24 h. The glass rods were washed by acetone and dried. The aminoalkylsilyl glass rods, thus prepared, were immersed in 1% aqueous solution of glutaraldehyde (Nakarai Chemicals Ltd, Kyoto) for 1 h. After washing with 0.25 M sodium phosphate buffer, pH 7.5, the glass rods were immersed in 0.25 M sodium phosphate buffer, pH 7.5, containing either non-specific or antibody IgG fractions from rabbit sera (1.0 to 2.0 mg/ml, unless otherwise specified) for 30 min and were stored at 4 °C overnight. The rabbit IgG-coupled glass rods were washed with 0.01 M sodium phosphate buffer, pH 7.0, containing 0.1 M NaCl, 1 mM $MgCl_2$, 0.1% NaN_3 and 0.1% bovine serum albumin (buffer A) and were immersed in buffer A for 2 h. Then, the glass rods were washed with buffer A and were stored in buffer A at 4 °C until use.

The Rabbit Antibody Fab' — β-D-galactosidase Complex

The rabbit antibody Fab' — β-D-galactosidase complexes were prepared using *N,N'-o*-phenylenedimaleimide (Aldrich Chemicals Company, Inc., Milwaukee) and β-D-glactosidase from *Escherichia coli* (Boehringer Mannheim, Mannheim) as described previously [7]. Amounts of the complexes are expressed as units of the enzyme activity, and one unit of the enzyme activity is defined as that which hydrolyzes one pmole of 4-methylumbelliferyl-β-D-galactoside (Sigma Chemical Co., St Louis) per min under the condition described below.

Sandwich Procedure

Each of the rabbit antibody IgG-coupled glass rods was incubated in a volume of 0.15 ml of buffer A, containing various amounts of antigen, with shaking at 37 °C for 4 h, unless otherwise specified, and the incubation mixture were allowed to stand at 4 °C overnight. Each rod was washed twice with 1 ml of buffer A, and was incubated with the rabbit antibody Fab' — β-D-galactosidase complex in a volume of 0.15 ml of buffer A with shaking at 37 °C for 6 h, unless otherwise specified. Each rod was then washed twice with 1 ml of buffer A and was transfered to another test tube to eliminate the non-specific binding of the complexes to the wall of test tubes, and β-D-galactosidase activity bound to each rod was assayed. Each rod was pre-incubated in 0.1 ml of buffer A at 30 °C for 5 min and the enzyme reaction was started by adding 50 µl of 0.3 mM 4-methylumbelliferyl-β-D-galactoside [16]. After 5 to 20 min of incubation at 30 °C with shaking 2.5 ml of 0.1 M glycine-NaOH buffer pH 10.3 were added and the amount of 4-methylumbelliferone (Nakarai Chemicals Ltd, Kyoto) formed was measured by fluorometry using Hitachi spectrofluorometer (model 204, Hitachi Ltd, Tokyo). Wave lengths used were 360 nm for excitation and 450 nm for emission analysis.

The sandwich immunoassay using the rabbit antibody IgG-coupled Sepharose 4B was performed as described previously [7].

The amounts of the complexes used for the assays of ornithine δ-aminotransferase from rat liver, human IgG, and 2,4-dinitrophenyl human IgG were 1650, 2000 and 2050 units, respectively, unless otherwise specified.

Temperature of Experiments

Unless otherwise specified, experiments were carried out at 20 to 25 °C.

RESULTS

Concentrations of Rabbit IgG for Coupling to Glass Rods

The glutaraldehyde-treated aminoalkylsilyl glass rods were treated with various concentrations of the rabbit anti(rat liver ornithine δ-aminotransferase) IgG fraction or non-specific rabbit IgG fraction, and the ability of the rabbit IgG-coupled glass rods, thus prepared, to serve for the sandwich immunoassay of ornithine δ-aminotransferase from rat liver was examined using the rabbit anti(rat liver ornithine δ-aminotransferase) Fab' — β-D-galactosidase complex. The results are shown in Table 1.

β-D-Galactosidase activities bound to the rabbit antibody IgG-coupled glass rods were increased with

Table 1. *Characteristics of the rabbit IgG-coupled glass rods prepared using various concentrations of rabbit IgG fractions*
The rabbit IgG-coupled glass rods prepared under indicated conditions were incubated with various amounts [(I) 0, (II) 0.068, (III) 0.68, (IV) 6.8 fmol] of ornithine δ-aminotransferase from rat liver, and then with the rabbit anti(rat liver ornithine δ-aminotransferase) Fab'–β-D-galactosidase complex. Values for β-D-galactosidase activity are the means of duplicate assays. Values in parentheses are the ratios of β-D-galactosidase activity bound to the glass rod in the presence of the antigen to that in its absence

Rabbit IgG fraction for coupling		β-D-Galactosidase activity bound to the glass rod			
specificity	concentration	I	II	III	IV
	(μg ml)	units rod			
Anti(rat liver ornithine δ-aminotransferase) IgG	2000	4.8 (1)	13 (2.7)	60 (13)	450 (94)
	200	6.1 (1)	14 (2.2)	72 (12)	458 (75)
	20	11 (1)	17 (1.5)	79 (7.2)	510 (46)
	2	7.6 (1)	13 (1.7)	73 (9.6)	257 (34)
	0.2	6.9 (1)	8 (1.2)	26 (3.8)	58 (8.4)
	0	4.0 (1)	–	5 (1.3)	5.5 (1.4)
Non-specific IgG	2000	4.8 (1)	–	–	7.7 (1.6)

Table 2. *Control of the relative amounts of the rabbit antibody IgG coupled to the glass rods*
The rabbit anti(rat liver ornithine δ-aminotransferase) IgG fraction was diluted to various degrees with the non-specific rabbit IgG fraction and was coupled to the aminoalkylsilyl glass rods at various concentrations (I, 2000 μg ml; II, 200 μg ml; III, 20 μg ml). The rabbit IgG-coupled glass rods were incubated with or without 6.8 fmol of ornithine δ-aminotransferase from rat liver and then with the rabbit anti(rat liver ornithine δ-aminotransferase) Fab'–β-D-galactosidase complex. Values for β-D-galactosidase activity are the means of duplicate assays. Values in parentheses are in percentage. β-D-Galactosidase activities bound without the antigen were 4.5 to 7.7 units for 2000 μg/ml, 5.1 to 8.9 units for 200 μg/ml, and 8.9 to 10.2 units for 20 μg/ml

Dilution with non-specific rabbit IgG	β-D-Galactosidase activity bound to the glass rod		
	I	II	III
-fold	units rod		
1	450 (100)	450 (100)	509 (100)
2	435 (97)	442 (101)	477 (94)
6	401 (89)	390 (90)	346 (68)
10	279 (62)	292 (67)	221 (43)
20	211 (47)	247 (57)	147 (29)
%	8	12	12

the increase of the antigen added, while β-D-galactosidase activities bound to the non-specific rabbit IgG-coupled glass rods or to the glass rods treated only with bovine serum albumin were not. This indicates that the complex was bound specifically to the corresponding glass rods through the bridge of the corresponding antigen, although the non-specific binding occurred to some extent. β-D-Galactosidase activities due to the specific binding were similar, when the glass rods were prepared using the concentration of 20, 200 or 2000 μg/ml of the rabbit antibody IgG fraction, but were decreased to a great extent, when they were prepared using the concentration of 0.2 or 2 μg/ml.

These observations indicate that the amount of the rabbit antibody IgG coupled to the glass rods is sufficient for the assay of less than 6.8 fmol of the antigen, when the glass rods are prepared with the concentrations of 20 to 2000 μg/ml. However, it is not necessarily implied that the same amount of the rabbit antibody IgG is coupled, when prepared with 20 to 2000 μg/ml.

The amount of rabbit IgG coupled could not be determined by measuring the change in the concentration of rabbit IgG fraction before and after coupling, since the change was too small to be determined precisely. So, relative amounts of the rabbit antibody IgG coupled to the glass rods which were prepared using different concentrations of the rabbit antibody IgG fraction were assesssed in the following manner. One volume of the solution of the rabbit anti(rat liver ornithine δ-aminotransferase) IgG fraction (20, 200 or 2000 μg/ml) was mixed with 1, 5, 9 or 19 vol. of the solution of non-specific rabbit IgG fraction (20, 200 or 2000 μg/ml), and the diluted antibody IgG fractions (20, 200 or 2000 μg/ml) were coupled to the aminoalkylsilyl glass rods. The amount of the IgG, specific for ornithine δ-aminotransferase from rat liver, coupled must have been reduced in proportion to the dilution. The rabbit antibody IgG-coupled glass rods thus prepared were tested by the sandwich procedure. As shown in Table 2, β-D-galactosidase activity bound to the glass rods was decreased by 3, 11, 38 and 53% when 2 mg/ml solution of the rabbit antibody IgG fraction for coupling was diluted 2-, 6-, 10- and 20-fold, respectively, with non-specific rabbit IgG fraction (2 mg/ml). When 200 μg/ml solution for coupling was diluted 2-, 6-, 10- and 20-fold, it was also similarly decreased by 0, 10, 33 and 43% respectively. These findings indicate that the amount of the rabbit antibody IgG coupled to the glass rods was not changed very much by lowering the concentration of the rabbit antibody IgG fraction for coupling from 2000 to 200 μg/ml. When 20 μg/ml solution was diluted 6-fold and was used for coupling, however, β-D-galactosidase activity bound to the glass rods was decreased by 32%. This decrease is similar to that when 200 or 2000 μg/ml solution was diluted 10-fold. These observations indicate that the amount of the rabbit antibody IgG coupled to the glass rods was

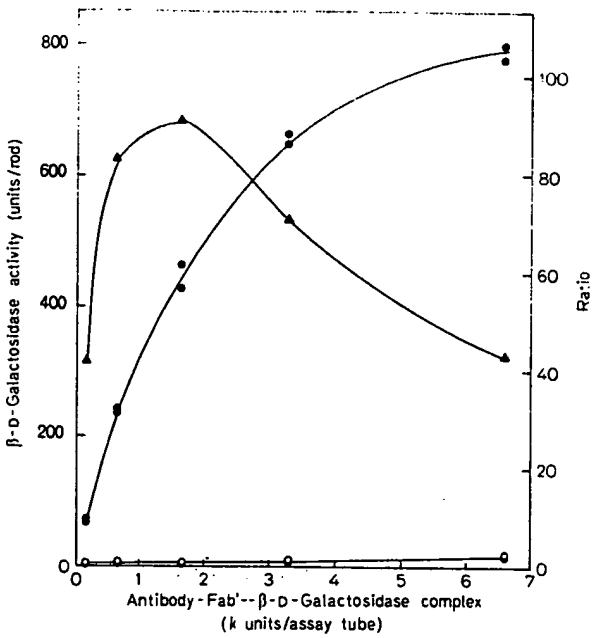

Fig. 1. *Optimal amount of the complex for the sandwich immunoassay.* The rabbit anti(rat liver ornithine δ-aminotransferase) IgG-coupled glass rods were incubated with (●) or without (○) 6.8 fmol of ornithine δ-aminotransferase from rat liver and then with various amounts of the rabbit anti(rat liver ornithine δ-aminotransferase) Fab'—β-D-galactosidase complex. (▲) The ratios of the mean values of duplicate assays with the antigen to those without it

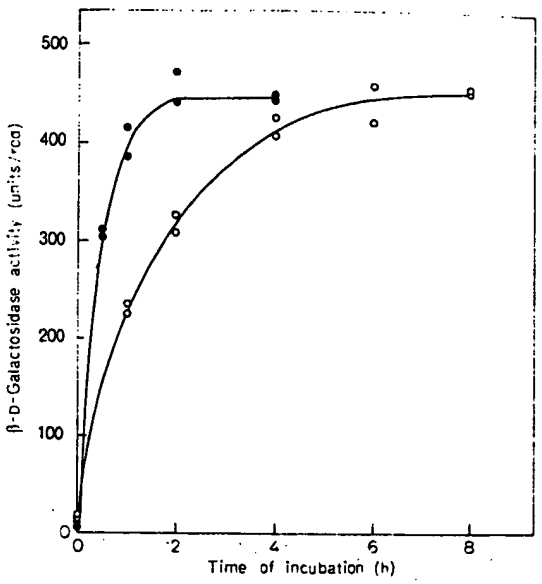

Fig. 2. *Periods of time required for the incubation with the antigen and with the complex.* The rabbit anti(rat liver ornithine δ-aminotransferase) IgG-coupled glass rods were incubated with ornithine δ-aminotransferase from rat liver for various periods of time ( ) or for 4 h (○) and then with the rabbit anti(rat liver ornithine δ-aminotransferase) Fab'—β-D-galactosidase complex for 6 h ( ) or for various periods of time (○)

reduced by less than 50% when 20 μg/ml solution was used for coupling.

The highest activity of β-D-galactosidase due to the non-specific binding was observed with the glass rods prepared using the concentration of 20 μg/ml, and the ratio of the enzyme activity due to the specific binding to that due to the non-specific binding was decreased as the concentration of the rabbit antibody IgG fraction for coupling was decreased (Table 1). This indicates that the glass rods prepared with higher concentrations of rabbit IgG fraction serve better for the immunoassay.

Optimal Amount of the Complex for the Assay

To assay ornithine δ-aminotransferase from rat liver with the optimal amount of the corresponding complex, the corresponding glass rods were incubated with or without 6.8 fmol of the antigen and then with various amounts of the corresponding complex. As shown in Fig. 1, both the specific and non-specific binding of the complex to the glass rods increased as the amount of the complex increased, but the ratio of β-D-galactosidase activity bound to the glass rod in the presence of the antigen to that in its absence was maximal when 1650 units of the complex were used. This amount of the complex should be optimal for the sandwich immunoassay of less than 6.8 fmol of the antigen.

Time Required for the Assay

The periods of time required for the incubation with the antigen and the complex in the assay of ornithine δ-aminotransferase from rat liver were examined. As shown in Fig. 2, the adsorption of the antigen on the corresponding glass rods reached the maximum within 4 h and the binding of the corresponding complex was almost maximal within 6 h.

Assay of Ornithine δ-Aminotransferase from Rat Liver

On the basis of the above findings, the sandwich immunoassay of ornithine δ-aminotransferase from rat liver was performed using the corresponding complex and glass rods. To compare with this, the assay of the antigen was also performed using the rabbit anti(rat liver ornithine δ-aminotransferase) IgG-coupled Sepharose 4B and the corresponding complex. As shown in Fig. 3, β-D-galactosidase activity due to the non-specific binding in the assay with the glass rods was about half of that with the Sepharose, despite that the amount of Sepharose 4B used was

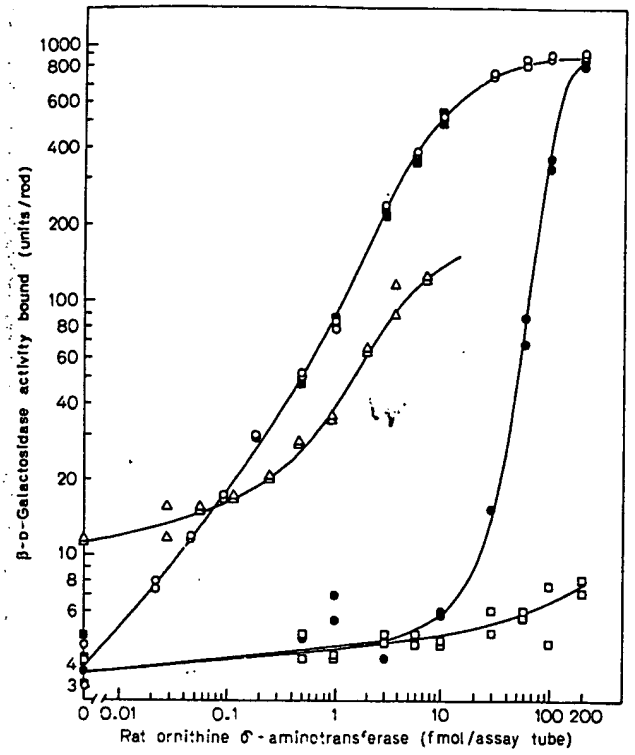

Fig. 3. *Assays of crystalline ornithine δ-aminotransferase from rat liver.* Crystalline ornithine δ-aminotransferase from rat liver was subjected to the sandwich immunoassay (the first assay) using the rabbit anti(rat liver ornithine δ-aminotransferase) IgG-coupled glass rods (○) or the non-specific rabbit IgG-coupled glass rods (□) and, after removing these glass rods, the incubation media were again subjected to the sandwich immunoassay (the second assay) with the rabbit anti(rat liver ornithine δ-aminotransferase) IgG-coupled glass rods. (●, ■) The second assays corresponding to the first assays indicated by open circles and squares, respectively. Open triangles indicate the sandwich immunoassay of the antigen using the rabbit anti(rat liver ornithine δ-aminotransferase) IgG-coupled Sepharose 4B

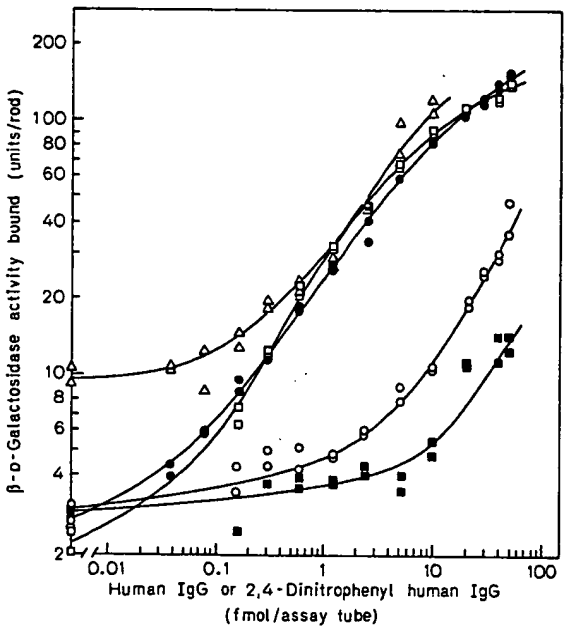

Fig. 4. *Assay of human IgG and 2,4-dinitrophenyl human IgG.* Human IgG was assayed with the corresponding complex and glass rods (△). 2,4-Dinitrophenyl human IgG was subjected to the (first) assay with the rabbit anti(human IgG) IgG-coupled glass rods (●) or with the non-specific rabbit IgG-coupled glass rods (■) and, after removing these glass rods, the incubation mixtures were again subjected to the (second) assay with the rabbit anti(human IgG) IgG-coupled glass rods. (○, □) The second assays corresponding to the first assays indicated by closed circles and squares, respectively. For 2,4-dinitrophenyl human IgG assay, the rabbit anti(2,4-dinitrophenyl) IgG-Fab′—β-D-galactosidase complex was used

reduced to the minimum that gave reproducible results [6,7]. This was caused in part by the fact that small quantities of Sepharose 4B particles could not be transfered from one test tube to another with reproducible results, while the glass rods could be easily transfered to eliminate the non-specific binding of β-D-galactosidase activity to the inner wall of test tubes. Consequently, 0.03 and 0.3 fmol of the antigen were measurable with the glass rods and the Sepharose 4B, respectively.

Assay of Human IgG and 2,4-Dinitrophenyl Human IgG

Human IgG was also subjected to the sandwich immunoassay using the rabbit anti(human IgG) IgG-coupled glass rods and the rabbit anti(human IgG) Fab′—β-D-galactosidase complex. As shown in Fig. 4, β-D-galactosidase activity due to the non-specific binding in this assay was much higher than that in the assay of ornithine δ-aminotransferase from rat liver. The smallest amount IgG that could be determined was 0.3 fmol in the assay shown in Fig. 4.

So, human IgG was not considered as an adequate model antigen for further improvements of the sandwich immunoassay, and 2,4-dinitrophenyl human IgG was prepared as a model antigen and was subjected to the sandwich immunoassay. The glass rods used were prepared using the rabbit anti(human IgG) or the rabbit anti(2,4-dinitrophenyl) IgG fraction. The rabbit anti(2,4-dinitrophenyl) Fab′—β-D-galactosidase complex was used for both assays with these glass rods. As shown in Fig. 4 and 5, β-D-galactosidase activities due to the non-specific binding in these assays were about one third of that in the assay of human IgG, and 0.04 and 5 fmol of dinitrophenyl human IgG could be determined using glass rods coupled with rabbit anti(human IgG) IgG and rabbit anti(2,4-dinitrophenyl) IgG, respectively. The preparation of 2,4-dinitrophenyl human IgG used con-

tained 8.3 2,4-dinitrophenyl residues per molecule and 2,4-dinitrophenyl human IgG containing 4.3 or 13 2,4-dinitrophenyl residues per molecule was assayed less sensitively.

Extent of the Adsorption of Antigens on the Rabbit Antibody IgG-Coupled Glass Rods

Ornithine δ-aminotransferase from rat liver and 2,4-dinitrophenyl human IgG were assayed with different sensitivities as mentioned above (Fig. 3–5). To understand the reason for this, the following experiments were performed. Various amounts of the antigens were subjected to the sandwich immunoassay (the first assay) and, after removing the glass rods used in the first assay, the incubation media were again subjected to the sandwich immunoassay (the second assay). 2,4-Dinitrophenyl human IgG was assayed using the rabbit anti(2,4-dinitrophenyl) Fab' –β-D-galactosidase complex and the glass rods coupled with rabbit anti(2,4-dinitrophenyl) or rabbit anti(human IgG) IgG. When the first assay was made using the rabbit anti(2,4-dinitrophenyl) IgG-coupled glass rods, the second assay was performed with the rabbit anti(human IgG) IgG-coupled glass rods as well as with the rabbit anti(2,4-dinitrophenyl) IgG-coupled glass rods. Results are shown in Fig. 3–5.

When smaller amounts of the antigens were assayed using the rabbit antibody IgG-coupled glass rods both for the first and second assays, β-D-galactosidase activities bound in all the second assays performed were similar to those due to the non-specific binding. When the non-specific rabbit IgG-coupled glass rods were used in the first assays, β-D-galactosidase activities bound in the second assay with the rabbit antibody IgG-coupled glass rods were similar to those in the first assay with the rabbit antibody IgG-coupled glass rods.

These findings indicate that smaller amounts of ornithine δ-aminotransferase and 2,4-dinitrophenyl human IgG to be assayed were almost completely adsorbed on the rabbit antibody IgG-coupled glass rods and that the difference in the sensitivities of the assays of these antigens was caused mainly by the difference in the ability of the antigens, adsorbed on the glass rods, to bind the complexes.

When larger amounts of the antigens were subjected to the first assays with the rabbit antibody IgG-coupled glass rods, β-D-galactosidase activities bound in the second assays with the rabbit antibody IgG-coupled glass rods increased gradually. When 100 fmol of ornithine δ-aminotransferase from rat liver were subjected to the first assay with the rabbit antibody IgG-coupled glass rods, β-D-galactosidase activity bound in the second assay with the rabbit antibody IgG-coupled glass rods was comparable to that in the first assay of 6 fmol of the antigen. After

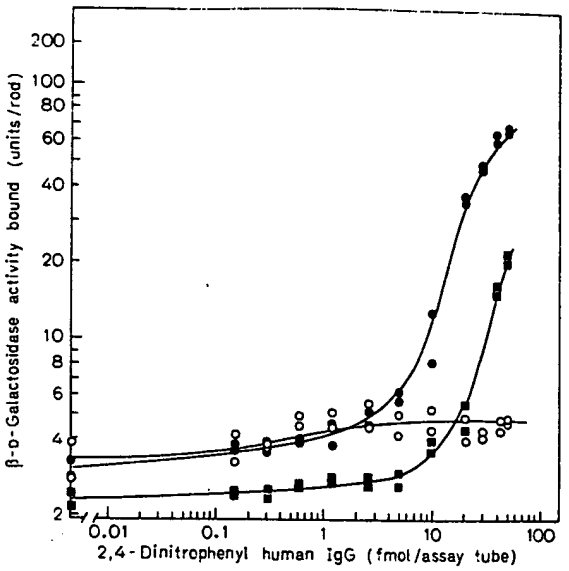

Fig. 5. *Assay of 2,4-dinitrophenyl human IgG.* 2,4-Dinitrophenyl human IgG was subjected to the (first) assay with the rabbit anti(2,4-dinitrophenyl) IgG-coupled glass rods (●) and then subjected to the (second) assay with the rabbit anti(2,4-dinitrophenyl) (○) or with the rabbit anti(human IgG) (■) IgG-coupled glass rods. The rabbit anti(2,4-dinitrophenyl) Fab' – β-D-galactosidase complex was used throughout

Table 3. *Recovery of rat liver ornithine δ-aminotransferase after the incubation with the non-specific rabbit IgG-coupled glass rods*
After indicated (initial) amounts of the antigen were subjected to the first assay with the non-specific rabbit IgG-coupled glass rods, indicated volumes out of 150 µl of the original incubation mixture, which had contained indicated (initial) amounts of the antigen, were subjected to the second assay with the rabbit anti(rat liver ornithine δ-aminotransferase) IgG-coupled glass rods. Total amounts of the antigen recovered in the second assay were calculated from the assay curve shown in Fig. 3

Initial amounts of antigen added	Volumes used for the 2nd assay	β-D-Galactosidase activity bound in the 2nd assay	Total amounts of antigen recovered in the 2nd assay
fmol	µl	units/rod	fmol
60	5.0	154	60
100	2.5	133	96
200	1.0	110	195

60 to 200 fmol of the antigen were subjected to the first assay with the non-specific rabbit IgG-coupled glass rods, aliquots of the incubation mixture were subjected to the second assay with the rabbit antibody IgG-coupled glass rods. As shown in Table 3, β-D-galactosidase activities bound in the second assays indicated that ornithine δ-aminotransferase from rat liver was not adsorbed on the non-specific rabbit IgG-coupled glass rods or on the inner wall of test tubes and that the amounts of the antigen added were recovered in the second assays. These findings indicate

that each of the rabbit anti(rat liver ornithine δ-aminotransferase) IgG-coupled glass rods was capable of binding at least 94 fmol of ornithine δ-aminotransferase from rat liver.

DISCUSSION

Cellulose [3] and Sepharose 4B [4–7] have been used as the solid phase for the sandwich immunoassay. To wash fine particles of cellulose or Sepharose 4B during the assay procedure, the supernatant fluid after centrifugation has to be removed carefully. When we used the rabbit antibody IgG-coupled Sepharose 4B (CNBr-activated) as a solid phase for the sandwich immunoassay of human IgG [4–7] and ornithine δ-aminotransferase from rat liver, values from duplicate assays were sometimes widely varied. This may be due in part to an inadequate washing of the Sepharose 4B. The glass rods are more easily washed and offer more reproducible results than Sepharose 4B. β-D-Galactosidase activity due to the non-specific binding in the assays with Sepharose 4B is higher than that in the assays with the glass rods (Fig. 1), and the assay with Sepharose 4B are less sensitive than those with the glass rods.

The procedure of washing the solid phase appears to be simplified to a great extent by using polystylene tubes [1,2]. However, it appears difficult to keep constant the non-specific binding of the complex to the solid phase by confining it to a certain limited area of the inner surface of polystylene tubes, especially when tubes are shaken for mixing incubation media. In contrast, the non-specific binding of the complex to the glass rods can be easily kept constant by transferring it into another test tube for the assay of enzyme activity after its incubation with the complex in one test tube. The glass rods are cheap and can be re-used after the treatment with 10 N NaOH and $K_2Cr_2O_7$-H_2SO_4 (unpublished).

The antibody-coupled glass rods may be applicable not only to the sandwich immunoassay but also to the competitive immunoassay of various antigens and haptens. For the latter purpose, coupling of limited amounts of antibodies to the solid phase is required. The findings in Table 2 indicate that it is possible to control the amounts of specific antibodies coupled to the glass rods by purifying antibody IgG or by diluting it with non-specific rabbit IgG. Such a control may also be possible by diluting with other proteins.

The amounts of the rabbit antibody IgG coupled to the glass rods is unknown. However, the findings in Fig. 3 may help to assess it. The data in Fig. 3 indicate that at least 94 fmol of ornithine δ-aminotransferase from rat liver was adsorbed on the individual glass rods. So, the amount of the rabbit antibody IgG, specific for the antigen, coupled to the individual glass rods was at least 47 fmol, since antibody IgG is divalent. The amount of IgG coupled must have been more than that, depending upon the purity of the rabbit antibody IgG fraction used.

To perform sensitive and accurate assays, the enzyme activity due to the non-specific binding of the complex to the solid phase should be as low as possible and that due to the specific binding should be as high as possible. The enzyme activity bound due to the specific binding must be proportional to the number of the complex bound to antigen which is adsorbed on the glass rod. The number of the complex bound specifically to individual molecules of ornithine δ-aminotransferase from rat liver adsorbed on the glass rods is calculated as follows. From the original activity of β-D-galactosidase preparation used, 53 units are calculated to correspond to 1 fmol of the enzyme. Assuming that the enzyme activity in the complex specifically bound to the glass rods was unaltered and was equal to the original activity, the amounts of the complex bound specifically to the glass rods are calculated by subtracting the enzyme activity due to the non-specific binding from the enzyme activity bound in the presence of the antigen and are expressed in fmol. The amounts in fmol of the complex thus calculated are plotted against the amount of the antigen assayed (Fig. 6). It is apparent that the number of the complex bound to individual antigen molecules on the rabbit antibody IgG-coupled glass rods is 2 or 3 when less than 0.4 fmol of the antigen were assayed using 1650 units of the complex and decreases gradually as the amount of the antigen to be assayed increased. However, its number was much smaller even with less than 0.4 fmol of the antigen, when 412 or 165 units of the complex were used. The number of the complex bound to 2,4-dinitrophenyl human IgG was apparently much less than that bound to ornithine δ-aminotransferase from rat liver (Fig. 3–5). Although the maximal number of the complex molecules that can be bound to individual antigen molecules on the rabbit antibody IgG-coupled glass rod remains to be determined, it may depend upon the molecular sizes of antigens to be assayed. So, higher sensitivities may be obtained for antigens of larger molecular sizes.

Glutaraldehyde-activated aminoalkylsilyl glass rods were treated with rabbit IgG fractions and then with bovine serum albumin, which was included in buffer A, to eliminate excess aldehyde groups on the glass rods. So, it is unlikely that aldehyde groups, remaining after coupling rabbit IgG, may limit the sensitivity of the present assay by raising the non-specific binding of the antigen proteins and/or the complexes to the glass rods. This is supported by the following findings. The aminoalkylsilyl glass rods were treated successively with glutaraldehyde, with non-specific rabbit IgG fraction (1.0 or 100 μg ml).

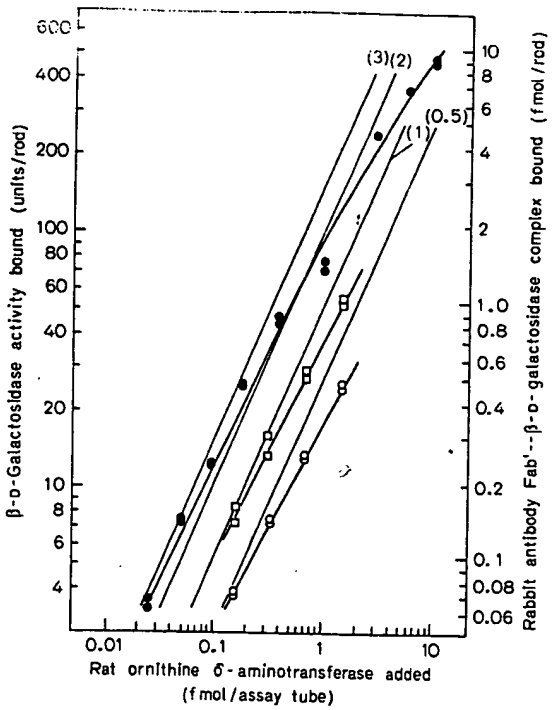

Fig. 6. *The number of the complex molecules bound to the individual antigen molecules adsorbed on the rabbit antibody IgG-coupled glass rods.* The number of the rabbit anti(rat liver ornithine δ-aminotransferase) Fab'−β-D-galactosidase complex molecules bound to the individual molecules of ornithine δ-aminotransferase from rat liver adsorbed on the rabbit anti(rat liver ornithine δ-aminotransferase) IgG-coupled glass rods is calculated from the data shown in Fig. 3, assuming that 53 units of the complex correspond to 1 fmol. The numbers of 0.5, 1, 2 and 3 in parentheses indicate the theoretical lines for the cases where the number of the complex molecules bound to individual antigen molecules on the glass rods is 0.5, 1, 2 and 3, respectively. The amounts of the rabbit antibody Fab'-enzyme complex used are 165 (○), 412 (□) and 1650 (●) units

with L-lysine (0.2 M L-lysine in 0.25 M sodium phosphate buffer (pH 7.5) at room temperature for 1 h and at 4 °C overnight) and finally with buffer A. For comparison, the glass rods were also prepared by omitting both treatments with glutaraldehyde and L-lysine or the treatment with L-lysine. The glass rods thus prepared were subjected to the sandwich procedure. The activities of β-D-galactosidase bound to these glass rods in the absence of antigen were all similar, whether the rabbit anti(rat liver ornithine δ-aminotransferase) or the rabbit anti(2,4-dinitrophenyl) Fab'−β-D-galactosidase complex was used. The activities of β-D-galactosidase bound to these glass rods following the incubation with ornithine δ-aminotransferase from rat liver (100 fmol) and then with the rabbit anti(rat liver ornithine δ-aminotransferase) Fab'−β-D-galactosidase complex were also all similar, although slightly higher than those in the absence of the antigen as shown in Fig. 3. When these glass rods were tested with 2,4-dinitrophenyl human IgG and the rabbit anti(2,4-dinitrophenyl) Fab'−β-D-galactosidase complex, the activities bound were also all similar, although about 6-fold higher than those in the absence of the antigen as shown in Fig. 4. In summary, the treatments with glutaraldehyde and/or L-lysine had little effect on the non-specific binding of the protein antigens and the complexes to the aminoalkylsilyl glass rods. The activity of β-D-galactosidase bound in the absence of antigen was relatively high when the glass rods were prepared using the concentration of 20 μg/ml of the rabbit IgG fraction (Table 1), and the reason for this is unknown.

The enzyme activity due to the non-specific binding of the complex in the assay of human IgG was higher than that in other assays and tended to increase with time of incubation. Frequently, remarkably high activities were observed as the non-specific binding. This may be due to the cross reaction between the rabbit IgG coupled to the glass rods and the rabbit anti(human IgG) Fab' fragment of the complex. This is supported by the fact that the enzyme activity due to the non-specific binding was low in the assay using the rabbit anti(human IgG) IgG-coupled glass rods and the rabbit anti(2,4-dinitrophenyl) Fab'−β-D-galactosidase complex.

The anti(2,4-dinitrophenyl) serum used might have contained the anti(bovine serum albumin) IgG, since the antiserum was produced using 2,4-dinitrophenyl bovine serum albumin. However, a possible interference with the assay would have been prevented by the presence of excess bovine serum albumin in the assay mixture. This is supported by the finding that the enzyme activities bound in the absence of the antigens were similarly low in the assay performed for 2,4-dinitrophenyl human IgG and ornithine δ-aminotransferase from rat liver (Fig. 3−5).

The maximal amount of antigens which can be assayed in the present system is limited by two factors. One is the capacity of the rabbit antibody IgG-coupled glass rods to bind antigens, and the other is the amounts of the complexes used. As described in Results, 94 fmol of ornithine δ-aminotransferase from rat liver were bound to the individual corresponding glass rods. Although the amount of the complex used was 1650 units, the maximal amount of the complex bound was 900 units (Fig. 3). This corresponds to 17 fmol, if it is assumed as mentioned above that 53 units correspond to 1 fmol. So, the factor to limit that maximal amount of the antigen that can be measured in the assay shown in Fig. 3 is the amount of the complex used. The proportion of the complex to bind specifically to the glass rods depends upon the purity of the antibody used and of the complex used [7].

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ENZYME-LINKED SANDWICH IMMUNOASSAY OF MACROMOLECULAR ANTIGENS USING THE RABBIT ANTIBODY-LOADED SILICONE PIECE AS A SOLID PHASE

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1. Introduction

Several procedures have been developed for the enzyme-linked solid phase sandwich immunoassay of macromolecular antigens [1–5]. We have recently developed a novel method for the conjugation of Fab′ fragments of rabbit immunoglobulin G (IgG) with β-D-galactosidase (EC 3.2.1.23) from *Escherichia coli* using *N,N′-o*-phenylenedimaleimide and have demonstrated that 0.3 fmol of a macromolecular antigen, human IgG, can be determined by the sandwich procedure using the purified rabbit antibody (Fab′)-β-D-galactosidase complex and the rabbit antibody (IgG)-coupled Sepharose 4B as a solid phase [6,7]. (Fab′: monovalent fragments derived from pepsin-treated IgG.) We describe a highly sensitive sandwich immunoassay of macromolecular antigens using the rabbit antibody (Fab′)-β-D-galactosidase complex and the rabbit antibody (IgG)-loaded silicone piece as a solid phase.

2. Materials and methods

Human IgG was obtained from Miles Laboratories, Inc. (Kankakee) and its amounts were determined from the absorbance at 280 nm [8]. 2,4-Dinitrophenyl(DNP)-human IgG was prepared using 2,4-dinitrobenzene sulfonic acid (sodium salt) (Tokyo Kasei Kogyo Co., Ltd., Tokyo) by the method of Eisen et al. [9]. The number of DNP residues coupled to human IgG and the amount of DNP-human IgG was calculated from the absorbance at 280 and 360 nm [8,9]. Rabbit anti-sera against human IgG and DNP-bovine serum albumin were obtained from Medical and Biological Laboratories Ltd. (Nagoya) and from Miles Laboratories, Inc. (Kankakee), respectively. IgG fractions from the anti-sera and non-specific rabbit serum were prepared by fractionation with Na_2SO_4 [10] followed by passage through a column of DEAE-cellulose (DE 52, Whatman Biochemicals Ltd., Kent) [11]. Crystalline ornithine δ-aminotransferase (EC 2.6.1.13) from rat liver and rabbit anti-sera against the crystalline enzyme were prepared as described previously [12]. The preparation of the crystalline enzyme was homogeneous on ultracentrifugation and electrophoresis and could be stored in 0.1 M sodium phosphate buffer, pH 7.5 without loss of its activity at −20°C for at least 6 months. Amounts in μg of the crystalline enzyme were determined by the method of Lowry et al. [13] using bovine serum albumin (Fraction V, Armour Pharmaceutical Co., Chicago) as a standard and its amounts in fmoles were calculated using its molecular weight of 170 000 [12]. IgG fraction from the anti-serum against the enzyme was obtained using $(NH_4)_2SO_4$ [14] and DEAE-cellulose [11].

2.1. *The rabbit antibody (Fab′)-β-D-galactosidase complexes*

The rabbit antibody (Fab′)-β-D-galactosidase complexes were prepared as described previously [7]. Their amounts are expressed as units of the enzyme activity, which are defined as described previously [7].

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2.2. *The rabbit antibody (IgG)-loaded silicone pieces*

Silicone tubes (2.5 mm in inner diameter and 4 mm in outer diameter; Silicone No.3, SH type, Fuji Kobunshi Kogyo Co., Ltd., Tokyo) were cut into pieces of 3 mm in length followed by longitudinal cut into half. The silicone pieces thus obtained were washed with a detergent (Scat 20-X, Nakarai Chemicals Ltd., Kyoto) followed by washing with water. They were then immersed in 0.25 M sodium phosphate buffer, pH 7.5, containing rabbit IgG fraction (20 to 2000 μg/ml) for 30 min and stored at 4°C overnight. They were then washed successively with 0.25 M sodium phosphate buffer, pH 7.5 and with 0.01 M sodium phosphate buffer, pH 7.0 containing 0.1 M NaCl, 1 mM $MgCl_2$, 0.1% NaN_3 and 0.1% bovine serum albumin (Buffer A) and stored in Buffer A at 4°C until use.

2.3. *Sandwich procedure*

The rabbit antibody IgG-loaded silicone pieces were incubated with various amounts of antigens in a final volume of 0.15 ml of Buffer A with shaking at 37°C for 4 h, and the incubation mixtures were allowed to stand at 4°C overnight. The silicone pieces were then washed twice with 1 ml of Buffer A and incubated with the rabbit antibody (Fab')-β-D-galactosidase complex in a volume of 0.15 ml of Buffer A with shaking at 37°C for 6 h. The silicone pieces were then washed twice with 1 ml of Buffer A and transfered into another test tube to eliminate the enzyme activity due to the non-specific binding of the complex to the wall of test tubes, and the enzyme activities bound to the silicone pieces were determined. The silicone pieces were pre-incubated in 0.1 ml of Buffer A at 30°C for 5 min and the enzyme reaction was started by adding 50 μl of 3×10^{-4} M 4-methylumbelliferyl-β-D-galactoside (Sigma Chemical Co., St. Louis). After 5 to 20 min of incubation at 30°C with shaking, the amounts of 4-methyl-umbelliferone formed were determined by fluorometry as described previously [7]. The amounts of the complexes used were 2050, 2000 and 1650 units for human IgG, DNP-human IgG and ornithine δ-aminotransferase from rat liver, respectively. These amounts were adjusted to the minimum that gave the maximal ratios of the enzyme activity bound in the presence of antigens to that in their absence.

The sandwich immunoassay using the rabbit antibody (IgG)-coupled Sepharose 4B was performed as described previously [7]. The amount of the complex used was equal to that in the assay with the silicone pieces.

2.4. *Temperature of experiments*

Experiments were carried out at room temperature (20 to 25°C) throughout, unless otherwise specified.

3. Results and discussion

Typical results of the sandwich immunoassays are shown in fig.1. In all the assays, the complexes corresponding to antigens were used. (The rabbit (anti-DNP) Fab'-enzyme complex was used for DNP-human IgG.) With the silicone pieces corresponding to antigens, 0.3 and 0.03 fmol of human IgG and ornithine δ-aminotransferase from rat liver, respectively, could be determined. With the rabbit (anti-DNP) IgG- or with the rabbit (anti-human IgG) IgG-loaded silicone pieces, 0.2 or 0.07 fmol of DNP-human IgG could be determined. The preparation of DNP-human IgG assayed contained 8.2 DNP residues per molecule, and the sensitivity was less for that containing 3.9 or 13 DNP residues per molecule. The (anti-DNP) serum used might have contained the (anti-bovine serum albumin) IgG, since the anti-serum was produced using DNP-bovine serum albumin. However, a possible interference would have been prevented by the presence of excess bovine serum albumin in the assay mixture. This is supported by the finding that the enzyme activities bound in the absence of antigens were similar in the assays performed for DNP-human IgG and ornithine δ-aminotransferase from rat liver (fig.1). The enzyme activity bound to the non-specific rabbit IgG-loaded silicone pieces did not increase with the increase of antigens added, indicating that the present assays are specific for each antigen.

Silicone pieces can be readily prepared in the laboratory and are much cheaper than polystylene tubes which have been used for the sandwich immunoassay [1,2]. The assays with the silicone pieces are less tedious than those with Sepharose 4B, which require washing by centrifugation [6,7].

When silicone pieces were loaded with rabbit antibody IgG by treating with the concentration of

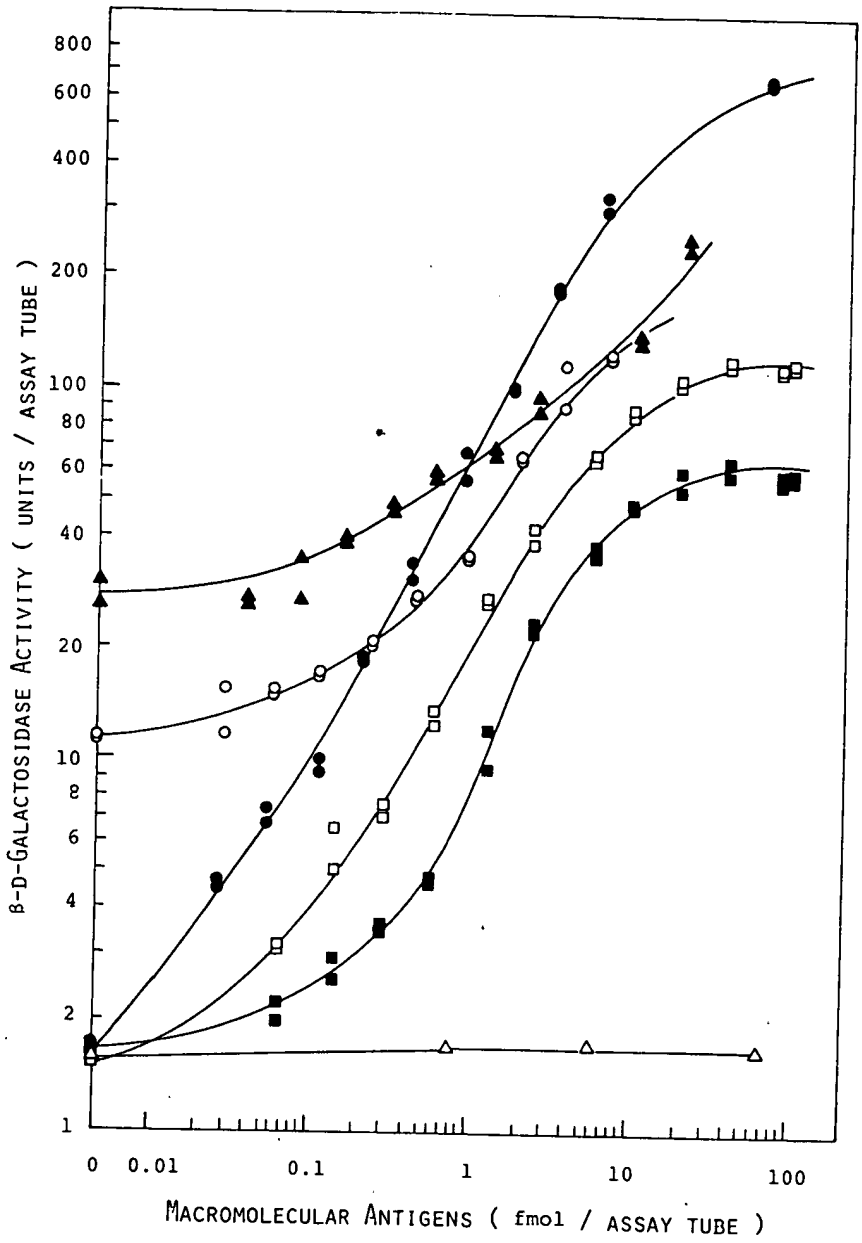

Fig.1. Solid phase sandwich immunoassays of macromolecular antigens. The solid phases used are: the rabbit (anti-human IgG) IgG-loaded silicone pieces for human IgG (closed triangles); the rabbit (anti-ornithine δ-aminotransferase from rat liver) IgG-loaded silicone pieces (closed circles), the rabbit (anti-ornithine δ-aminotransferase from rat liver) IgG-coupled Sepharose 4B (open circles) or the non-specific rabbit IgG-loaded silicone pieces (open triangles) for ornithine δ-aminotransferase from rat liver; the rabbit (anti-DNP) (closed squares) or the rabbit (anti-human IgG) (open squares) IgG-loaded silicone pieces for DNP-human IgG. All the silicone pieces used were prepared by treating with the concentrations of 1.0 mg/ml of the corresponding IgG fractions. In all the assays performed, the rabbit antibody (Fab')-β-D-galactosidase complexes corresponding to antigens were used. (The rabbit (anti-DNP) Fab'-enzyme complex was used for DNP-human IgG.)

20, 200 or 2000 μg/ml of rabbit antibody IgG fractions, the enzyme activities bound in the presence of antigens were not affected by the concentrations used. After the rabbit antibody (IgG)-loaded silicone pieces were washed up to 5 times by shaking in Buffer A or in 0.25 M sodium phosphate buffer, pH 7.5 containing non-specific rabbit IgG fraction (2 mg/ml) or bovine serum albumin (10 mg/ml) at 37°C for 4 h, they showed the same ability to bind the rabbit antibody (Fab')-β-D-galactosidase complexes in the presence of antigens as before washing. These findings indicate that a wide range of the concentrations of rabbit IgG is effective for its stable loading, although its amount loaded remains to be determined.

The non-specific binding in the assay with silicone pieces was about one seventh of that with Sepharose 4B, even if the amount of Sepharose 4B used was reduced to the minimum that gave reproducible results. This may be due in part to the fact that small quantities of Sepharose 4B can not be transferred from one test tube to another with reproducible results, while the silicone pieces can be easily transferred. With Sepharose 4B, 0.2 fmol of ornithine δ-aminotransferase from rat liver could be determined, while 0.03 fmol could be determined using the silicone pieces (fig.1). The non-specific binding in the assay of human IgG was much higher than those in the assay of other antigens and tended to increase with time of incubation. This may be due to the cross reaction between rabbit IgG on the solid phase and rabbit (anti-human IgG) Fab' of the complex. DNP-human IgG, not human IgG, may be an adequate model antigen for further improvements of the assay, since DNP-human IgG and antibody against it are readily prepared and commercially available, respectively.

The assay of DNP-human IgG with the rabbit (anti-DNP) IgG-loaded silicone pieces was less sensitive than that with the rabbit (anti-human IgG) IgG-loaded silicone pieces (fig.1.), although the same amount of the complex was used for both assays. To understand the reason for this, various amounts of the antigen (less than 20 fmol) were subjected to the sandwich immunoassay using the (anti-DNP) or the (anti-human IgG) IgG-loaded silicone pieces (first assay) and, after removing those pieces, the incubation media were again subjected to the sandwich assay (second assay). The enzyme activities bound in the second assay for both assays were similar to those in the absence of the antigen, indicating that the antigen to be assayed was completely adsorbed in the first assay for both assays. (When the non-specific rabbit IgG-loaded silicone pieces were used in the first assay, the enzyme activities bound in the second assay were equal to those in the first assay with the rabbit antibody (IgG)-loaded silicone pieces.) This indicates that the sensitivities in the present assay of DNP-human IgG depend upon the ability of the antigen, adsorbed on the solid phase, to bind the complex but not upon the efficiency of adsorption.

Our preliminary experiments suggested that the antibody (IgG)-loaded silicone pieces are applicable not only to the sandwich immunoassay but also to the competitive immunoassay of antigens and haptens and the assay of antibodies.

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